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X-ray Grade Crystals of the Enzymatic Fragment of Diphtheria Toxin*

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The enzymatic fragment of diphtheria toxin, fragment A (Mr = 21,167), complexed to the dinucleotide adenosine 3′,5′-uridine (ApU), has been crystallized at two different values of pH by hanging drop vapor diffusion. Crystals grown at a pH value of 5.0 (from I) belong to the orthorhombic space group $P2_12_12_1$, with unit cell parameters a = 71.2 Å, b = 73.0 Å, c = 139.8 Å and four protomers in the asymmetric unit. Crystals grown at a pH value of 8.1 (form II) belong to the monoclinic space group C2, with unit cell parameters a = 65.2 Å, b = 85.6 Å, c = 34.6 Å, β = 103.0° and one protomer in the asymmetric unit. Both crystal forms diffract to 2.5 Å resolution. The molecular structures of fragment A obtained from these two crystal forms may illuminate the pH-dependent transition of diphtheria toxin during membrane translocation.

Translocation of proteins across cell membranes is a topic of intense investigation. Formidable energetic barriers must be overcome in the movement of highly charged, hydrophilic, high molecular weight molecules such as proteins across the lipid bilayer. Although the molecular mechanics for this movement are not well understood, it is one view that there is a common mechanism for all translocations of proteins across membranes which, with minor modifications, can explain the movement of a diversity of proteins (1).

Toxins made by pathogenic bacteria offer us the opportunity to study the mechanisms of protein transport. These toxins, which are capable of translocating from the cell surface into the cytosol, include diphtheria toxin (2), shigella toxin (3), cholera toxin (4), *Pseudomonas* endotoxin A (5), tetanus toxin (6), and botulinum toxin (6). Each of these toxins is believed to have an enzymatic moiety, called the A fragment, whose substrate lies in the cytosol. The remainder of the molecule, the B fragment, binds to receptors on the cell surface and facilitates the transport of the A fragment across the plasma membrane into the cytosol.

Diphtheria toxin is one of the most extensively studied of these toxins (7), and it has received much attention because of its use in the preparation of immunotoxins (8). Designed for targeted cell killing in cancer and other diseases, these molecules consist of the toxin or fragments thereof, attached to antibodies against certain molecular markers on the cell surface. Such immunotoxins have not been very effective in fighting disease, perhaps because the relationship between the molecular structure of the toxin and its ability to enter the target cell is not well understood.

Diphtheria toxin (DT)[1] is a protein produced by *Corynebacterium diphtheriae* that has been infected with the β *tox* phage. The toxin is secreted from the bacterium as a single polypeptide chain, 535 residues in length, for which the amino acid sequence has been inferred (9). The polypeptide chain contains two disulfide bonds. Whole toxin is readily proteolyzed at an arginine-rich region within the first disulfide loop, yielding a two-fragment structure linked by a disulfide bond. Reduction of this disulfide produces two fragments: A (N-terminal, M_r = 21,167) and B (C-terminal, M_r = 37,195) (10).

The A fragment is an enzyme that catalyzes the NAD^+-dependent transfer of an ADP-ribose moiety to elongation factor 2 (EF-2) (5) (Equation 1).

$$NAD^+ + ADP - ribose + EF - 2 \rightleftharpoons ADP - ribosyl\ EF - 2 + nicotinamide + H^+ \quad (1)$$

This EF-2 derivative is inactive in promoting the translocation event on ribosomes. Peptide elongation is blocked, eventually causing cell death. The equilibrium for the ADP-ribosylation of EF-2 by fragment A lies far to the right at neutral pH.

Several studies have demonstrated that DT enters cells via receptor-mediated endocytosis, and that the molecule is transported to the cytoplasm by penetration of the membrane of an acidic organelle, such as an endosome (11–13). The B fragment, which has no apparent enzymatic activity, but which is required for toxicity (14), interacts with the lipid bilayer. It binds to external plasma membrane receptors (7, 15), after which membrane penetration is probably triggered by a low pH-induced conformational change in the toxin (16, 17). Fragment B then inserts into and crosses the endosomal membrane, facilitating the transport of fragment A, which also undergoes some change of conformation at low pH (18), to its target substrate in the cytosolic compartment.

The A fragment has a single NAD-binding site, which stabilizes the molecule against proteolytic attack (10). Complications arise in attempting to crystallize fragment A with bound NAD, because fragment A does show weak ADP-ribosylation activity and NAD glycohydrolase activity (10). The dinucleotide adenosine 3′,5′-uridine (ApU), however, is an effective ligand, believed to bind at the NAD site on fragment A. While previous attempts at crystallizing fragment

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[1] The abbreviations used are: DT, diphtheria toxin; EF-2, elongation factor 2; ApU, adenosine 3′,5′-uridine.

A in the absence of NAD were not successful, our addition of ApU has produced crystals.

We are interested in determining the structure of fragment A in several forms to understand better how this hydrophilic, high molecular weight molecule is able to cross the lipid bilayer at low pH and then assume its active conformation in the cytosol at high pH. In addition, elucidation of these structures may aid in the design of efficient cell-specific anticancer agents, such as immunotoxins. A partial three-dimensional structure of whole diphtheria toxin at a pH of 8.0 has been determined at UCLA (19, 20). We report here the crystallization of the catalytic fragment of diphtheria toxin, fragment A, at pH values of 5.0 and 8.1, bound to ApU.

EXPERIMENTAL PROCEDURES

Purified fragment A was prepared according to the methods of Carroll and co-workers (21). Approximate crystallization conditions for the protein were determined at the outset of the experiments using the incomplete factorial method (22, 23).

Crystals of the nucleotide-bound fragment, referred to as form I, were grown at room temperature by vapor diffusion (24). A 10% molar excess of ApU (Sigma) was added to a solution containing 20–25 mg/ml of fragment A in 50 mM Tris-HCl, pH 7.5. A volume of 10 μl of the protein solution was mixed with an equal volume of mother liquor containing 75 mM magnesium chloride, 100 mM sodium acetate buffer, pH 5.0, and 20% polyethylene glycol 4000 (Sigma). The resulting mixture was suspended from a siliconized microscope slide and sealed over a 1.5-ml reservoir of the mother liquor in a Linbro plate. Crystals appeared in 4–5 days and grew to full size in 7–8 days. They were mounted for diffraction studies directly from their mother liquor and were characterized using standard crystallographic methods (25) on a Nonius precession camera and an Elliott GX6 rotating anode generator operating at 1.6 kW as the x-ray source.

Crystals of nucleotide-bound fragment A of form II, were also grown by hanging drop vapor diffusion. A 10% molar excess of ApU was added to a solution containing 20–25 mg/ml of fragment A in 50 mM Tris-HCl, pH 7.5. A volume of 10 μl of the protein solution was mixed with an equal volume of mother liquor containing 0.45 M lithium acetate, 25 mM Tris-HCl, pH 8.1, and 30% polyethylene glycol 8000 (Sigma). The resulting mixture was suspended from a siliconized microscope slide and sealed over a 1.5-ml reservoir of the mother liquor in a Linbro plate. Crystals appeared in 3–4 days and grew to full size in 6–7 days. They were characterized in the same manner described above for form I.

RESULTS

A summary of the refined crystallization conditions, crystal properties and cell parameters are given in Table I. Precession photographs of two zones located 90° apart, as well as subsequent precession photographs, showed that the form I crystals of fragment A belong to the orthorhombic space group $P2_12_12_1$, with a unit cell volume of 7.27×10^5 Å^3 and unit cell parameters $a = 71.2 \pm 0.2$ Å, $b = 73.0 \pm 0.2$ Å, $c = 139.8 \pm 0.4$ Å. On the basis of unit cell volume, and assuming four protomers in the asymmetric unit, the calculated Matthews number, V_M, had a value of 2.2 Å^3, giving a protein content for the unit cell of 57% by weight, typical of protein crystals (26). The crystals diffracted well, but anistopically with measurable intensities to 2.5 Å resolution in the *b* and *c* directions and 3.0 Å in the *a* direction. They are stable for at least 6 months. Native data and data for two heavy atom derivatives have been collected by area detector methods (27, 28). The self-rotation function (29) revealed that the non-crystallographic 4-fold axis is parallel to the *b* axis of the cell.

Similar precession methods revealed that form II crystals of fragment A belong to the monoclinic space group C2, with a unit cell volume of 1.88×10^5 Å^3 and unit cell parameters $a = 65.2 \pm 0.2$ Å, $b = 85.6 \pm 0.3$ Å, $c = 34.6 \pm 0.1$ Å, $\beta = 103.0° \pm 0.2°$. The assumption of one protomer in the asymmetric unit gave a value for V_M of 2.2 Å^3, which yields the same percentage by weight of protein described above. The crystals showed diffraction having measurable intensities to 2.5-Å resolution. Crystals that were not disturbed for 3–4 weeks lost much of their high resolution diffraction pattern, showing marginal intensities to 3.8-Å resolution. Collection of native data and the search for heavy atom derivatives have been initiated.

DISCUSSION

Using the diffraction quality crystals of fragment A of diphtheria toxin, we hope to determine its molecular structure

TABLE I

Properties and growth conditions for three crystal forms of fragment A

Crystal form	ApU	Morphology	Size	Space group	Unit cell dimensions	Number of protomers/ asymmetric unit	V_M	d_{min}	Growth conditions[a]
			mm					*Å*	
I	+	Long arrows	2.5	$P2_12_12_1$	$a = 71.2 \pm 0.2$ Å	4	2.2	2.7	75 mM Magnesium chloride
			×0.5	(orthorhombic)	$b = 73.0 \pm 0.2$ Å				100 mM Sodium acetate, pH 5.0
			×0.5		$c = 139.8 \pm 0.4$ Å				20% Polyethylene glycol 4000
II	+	Oblique needles	1.2	C2	$a = 65.2 \pm 0.2$ Å	1	2.2	2.5	0.45 M Lithium acetate
			×0.2	(monoclinic)	$b = 85.6 \pm 0.3$ Å				25 mM Tris-HCl, pH 8.1
					$\beta = 103.0 \pm 0.2°$				
			×0.2		$c = 34.6 \pm 0.1$ Å				30% Polyethylene glycol 8000
III	−	Plates	0.7						25 mM Magnesium chloride 50 mM Sodium acetate, pH 5.3 20% Polyethylene glycol 4000

[a] The concentrations of polyethylene glycol indicated are expressed as weight/unit volume. The pH given is that of a 1.0 M stock solution of buffer.

and to begin to elucidate the pH-dependent mechanisms of cellular entry by diphtheria toxin. Although it is still unknown if the conformation of DT-A in forms I and II is in fact different, differences in structure of DT-A in the two crystal forms could give direct information about the proposed pH-induced conformational changes (16, 18, 30). The active conformation, which may be provided by the structure of form II, will help to explain the mechanism of ADP-ribosylation, as well as provide structural information that can be incorporated into the architecture of immunotoxins (8).

Although we have no direct evidence that either form I or form II contains ApU, a complex might be expected on the basis of binding constants. Collins and Collier (10) measured a K_I value of 22 μM for ApU binding to DT-A at 37 °C, pH 7.1, far below the concentration of ApU of about 1 mM in our crystallization experiments. (They did not explore the pH dependence of ApU binding.) Thus it seems likely that ApU is present in the crystals. This supposition is supported by our repeated failure to grow diffraction quality crystals of DT-A in the absence of ApU.

An electron density map of a dimer of nucleotide-bound whole diphtheria toxin (fragments A and B) at a pH value of 8.0 has been calculated and partially interpreted to 3.0-Å resolution (20). Problems that have hindered the calculation of a fully interpretable map are discussed elsewhere (19, 20). In view of these difficulties, we would like to exploit the method of molecular replacement (31) using phase information from the structure of fragment A at pH 8.1 to improve the current phase information for the electron density of the whole toxin, so that its complete three-dimensional structure can be unambiguously determined.

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